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A tomato alternative oxidase protein with altered regulatory properties

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Abstract

We have investigated the expression and regulatory properties of the two alternative oxidase (Aox) proteins that are expressed in tomato (*Lycopersicon esculentum* L. Mill cv. Sweetie) after storage of green fruit at 4 °C. Four *Aox* genes were identified in the tomato genome, of which two (*LeAox1a* and *LeAox1b*) were demonstrated to be expressed in cold-treated fruit. The activity and regulatory properties of LeAox1a and LeAox1b were assayed after expression of each protein in yeast cells (*Saccharomyces cerevisiae*), proving that each is an active Aox protein. The LeAox1b protein was shown to have altered regulatory properties due to the substitution of a Ser for the highly conserved Cys_I residue. LeAox1b could not form inactive disulfide-linked dimers and was activated by succinate instead of pyruvate. This is the first example of a dicot species expressing a natural Cys_I/Ser isoform. The implications of the existence and expression of such Aox isoforms is discussed in the light of the hypothesised role for Aox in plant metabolism.

Keywords: Alternative oxidase; Tomato; Mitochondria; Diagonal PAGE

1. Introduction

The alternative oxidase (Aox) is part of the branched respiratory electron transport chain (ETC) that is present in all plant mitochondria studied to date, as well as many fungal and some protist mitochondria. Aox accepts electrons from the ubiquinone (UQ) pool and reduces oxygen to water, but does not translocate protons and therefore is not subject to respiratory control by adenylates. There has been increased interest in this enzyme recently due to its apparent role in protecting plant mitochondria from reactive oxygen species (ROS) [1–7]. This protective function is hypothesised to be due to the ability of the oxidase to catalyse non-coupled respiratory electron transport, thus maintaining ETC components in a more oxidised state and decreasing ROS formation by the ETC [8].

The non-coupled electron transport catalysed by Aox is highly regulated at a number of levels to prevent wasteful respiration [9–11]. The plant Aox exists as a dimer whose subunits can be covalently linked through the formation of a disulfide bond [12]. The reduced dimer is active in the presence of keto acids such as pyruvate [13], while the oxidised, covalently linked dimer is inactive. When activated, Aox can compete with complex III for electrons from UQH_2 and thereby potentially alter the rate and efficiency of respiration [14]. Site-directed mutagenesis studies have identified a highly conserved Cys residue (termed Cys_I; [15]) as the site of both redox and keto acid regulation [16–19]. In the latter case, Cys_I has been suggested to form a thiohemiacetal with pyruvate [20]. A second highly conserved Cys residue (Cys_{II}) also appears to influence the activity of the enzyme but its role has not been clearly determined [16,17,19]. In vivo, reduction of Aox proteins probably occurs via mitochondrial NADPH pools linked to mitochondrial specific isoforms of thioredoxin and thioredoxin reductase [21,22]. The two mechanisms of activation provide an attractive feed-forward regulatory mechanism which ensures that Aox will be active when carbon flow through mitochondria is rapid (and the potential for ROS formation highest), and inactive when carbohydrate levels are low. Superimposed on this post-translational control is transcriptional control in response to various stress conditions, metabolic retrograde feedback and developmental programs [23–25].

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Since the identification of the regulatory Cys residues, a number of naturally occurring Aox isoforms have been identified which contain Cys to Ser substitutions at one or both of these sites, including four monocot Aox sequences (TC48674, *Hordeum vulgare* TIGR databases; TC69262, TC69263 *Triticum aestivum* TIGR databases; *Oryza sativa*, [26]; *Zea mays*, [27]). Investigations into the expression and activity of such isoforms will increase the understanding of the in vivo regulation of respiration. Indeed, Karpova et al. [27] have reported differential expression in maize of the normal and "Cys minus" Aox isoforms in independent non-chromosomal stripe mutants and in response to various ETC inhibitors. In particular, the "Cys-minus" isoform, ZmAox3, was found to be expressed in response to ETC complex IV dysfunction, while the Cys-containing ZmAox2 isoform was induced by complex I dysfunction. However, no biochemical studies of these enzymes have been reported and their response to activation mechanisms remains unclear.

During studies into the role of Aox in tomato fruit ripening and cold storage [28], we have amplified and sequenced two full-length *Aox* cDNAs, including one with a Cys_I to Ser substitution. We have investigated the expression and regulation of both proteins in tomato plants and by heterologously expressing the proteins in yeast, demonstrating for the first time that a naturally occurring Cys to Ser isoform is expressed in wild-type plant tissues and is active in vivo.

2. Materials and methods

2.1. Materials

Tomato (*Lycopersicon esculentum* L. Mill cv. Sweetie) seed was purchased from Yates (Sydney, NSW, Australia). Soybean seeds (*Glycine max* L. cv. Stevens) were obtained from NSW Agriculture (Narrabri, Australia). The AOA monoclonal antibody raised against *Sauromattum guttatum* Aox [29] was a generous gift from Dr. T. Elthon (University of Nebraska, USA). The *Saccharomyces cerevisiae–Escherichia coli* shuttle vector, pYES3, was obtained from Dr. F. Smith (CSIRO, Brisbane, Australia) [30]. *S. cerevisiae* strain R757 was obtained from Dr. R. Graber (Northwestern University, Illinois, USA).

2.2. Plant and yeast culture

Tomato plants were grown in 30-cm diameter pots containing commercial potting mixture in a naturally illuminated glasshouse. Tomato flowers were allowed to self-pollinate and mature green tomato fruits were harvested as required and stored at 4 °C for 1 week before use. Soybean seedlings were grown in damp vermiculite in growth cabinets with a 14 h–10 h light–dark cycle at 28 °C, 65% relative humidity.

Yeast transformations were performed according to Gietz and Schiestl [31] using the *S. cerevisiae* strain R757 (MATα, his 4–15, lys 9, ura 3–52). The *LeAox1a* and *1b* cDNAs were amplified from the pGEM-T plasmid using primers designed to allow directional insertion into the yeast transformation vector pYES.

LeAox1a *Bam*H1: 5′ GGA TCC CAG ATT TTG ATC GG 3′
LeAox1a *Sph*I: 5′ GCA TGC TAC TCT ACC TCT TAT CG 3′
LeAox1b *Eco*R1: 5′ GAA TTC GAA TAT GCC TCG TAA TGC 3′
LeAox1b *Sph*I: 5′ GCA TGC GAT ATT GTT TTC ACT TC 3′

Transformants were selected on SD medium agar plates (0.67% (w/v) yeast nitrogen base, 2% (w/v) D-glucose, 0.002% (w/v) each, L-tryptophan, L-histidine, L-methionine, L-lysine and adenine, 0.003% (w/v) L-leucine, 2% (w/v) agar) at 28 °C. Expression of LeAox proteins was induced by the substitution of 2% (w/v) galactose for 2% (w/v) glucose in the SD medium. For mitochondrial isolation, yeast cells grown overnight in SD-glucose medium with 200 rpm shaking were washed with SD medium containing 2% (w/v) galactose and used to inoculate fresh SD-galactose medium. The fresh cultures were incubated at 28 °C, 200 rpm for 5–6 days until OD_{600} was at least 5.

2.3. Standard nucleic acid methods

Nucleic acids were isolated according to standard protocols [32]. Degenerate oligonucleotide primers designed to amplify a highly conserved 170-bp fragment of plant Aox genes [33] were used in PCR reactions with tomato genomic DNA as the template. The cycling conditions were 95 °C, 2 min; (95 °C, 15 s; 47 °C, 15 s; 72 °C, 2 min) × 30 cycles; 72 °C, 5 min; 22 °C, 6 min; hold at 4 °C. PCR amplifications were performed using a thermocycler with a gradient block (Mastercycler, Eppendorf). An adaptor ligated cDNA library was generated from $polyA^+$ RNA isolated from the pericarp of chilled green tomato fruit according to the kit manufacturer's instructions (Marathon Ready cDNA kit, Clontech, CA, USA). Gene-specific primers were designed against tomato *Aox* sequences determined in this study, and used in 5′ and 3′ rapid amplification of cDNA ends (RACE) reactions with an annealing temperature of 48 °C. Note that only a 5′ RACE primer was needed to amplify the full-length clone of LeAox1b, because the 3′ end, including the stop codon, had been isolated previously from a cDNA library from ripening fruit (Holtzapffel, Finnegan and Day, unpublished results). The sequence of the adaptor primer (AP1, Clontech) used in all RACE reactions was:

5′ CCA TCC TAA TAC GAC TCA CTA TAG GGC 3′.
The sequences of the gene-specific RACE primers were:

LeAox1a 5′ RACE, 5′ GGG GAA ATA AGG TAT GCA GC 3′,
LeAox1a 3′ RACE, 5′ GAT GAC TTT CAT GGA AGT TGC 3′,
LeAox1b, 5′ RACE, 5′ GGA GAT ATT GTT TTC ACT TCA 3′.

Amplification products were separated on agarose gels, and the desired fragments excised and purified using a commercial kit (Wizard PCR preps Direct purification System, Promega) before ligating into a T-tailed vector (pGEM-T, Promega).

A full-length *LeAox1a* cDNA was produced by fusing overlapping 5′ and 3′ RACE products ligated in pGEM-T at the unique *Acc*I site internal to the cDNA. DNA sequencing reactions were performed using dye-terminator chemistry (BigDye, Applied Biosystems) and analysed using a DNA sequencer (Genetic Analyser, ABI Prism, Applied Bioscience). Sequence analysis was performed using the GCG suite of programs accessed through the Australian National Genomic Information Service (http://www.angis.su.oz.au). The BLAST algorithm was used to search sequence databases for sequence similarity through the National Centre for Biotechnology Information site (http://www.ncbi.nlm.nih.gov).

2.4. Competitive reverse transcription-PCR

Total RNA was reverse transcribed using the MMLV-RT enzyme (Clontech) according to the manufacturer's instructions. Homologous fragments of *LeAox1a* and *1b* cDNAs were amplified simultaneously and competitively using a single PCR primer pair designed to anneal at highly conserved sites on cDNAs from both genes. The forward primer contained two sites of degeneracy to compensate for differences in the annealing sites between *LeAox1a* and *1b*, while the reverse primer sequence was present in both cDNAs. The sequences of the primers were:

Forward: 5′ GYT TYA GGC CAT GGG AGA C 3′
Reverse: 5′ CGA TGA GCC AAT TTT GGG G 3′

An annealing temperature of 53 °C was used. The products from *LeAox1a* and *1b* cDNA could be differentiated by digestion with *Csp*45I and *Rca*I, respectively.

2.5. Isolation of mitochondria

Tomato fruit mitochondria were isolated according to the method of Holtzapffel et al. [28]. Soybean mitochondria were isolated from 7-day-old cotyledons according to the method of Day et al. [34]. Yeast mitochondria were isolated using the method of Pratje and Michaelis [35] and purified by centrifugation through a 21% self-generating Percoll gradient in lysis buffer, at 40,000 × *g* for 40 min. The uppermost band containing the mitochondria was removed from the gradient and washed free from Percoll by centrifugation at 15,000 × *g* for 30 min. The final pellet was resuspended in standard reaction buffer (see below).

2.6. Assays

Oxygen consumption was measured with a Clark-type O_2-electrode (Rank Bros., Cambridge, UK). Samples of mitochondria were suspended in 1 ml reaction medium (0.3 M sucrose, 5 mM KH_2PO_4, 10 mM TES, pH 7.0, 10 mM NaCl, 2 mM $MgSO_4$, 0.1% (w/v) BSA) at 25 °C. Subsequent additions were made as detailed in the Results section.

In vitro import of LeAox proteins was performed as described [36], using ^{35}S-methionine-labelled precursor proteins produced in a coupled transcription-translation rabbit reticulocyte system (TNT, Promega, WI, USA).

The concentration of protein in mitochondrial samples was determined by a modified Lowry method [37]. Some samples were treated with diamide for 30 min at RT prior to solubilisation in loading buffer lacking reductant and these were not heated. Yeast mitochondrial membrane samples destined for SDS-PAGE were treated with a protease inhibitor cocktail (Complete™, Roche Diagnostics, Mannheim Germany) before incubation with diamide or DTT.

2.7. Diagonal PAGE

In the first dimension, 40 or 300 μg mitochondrial protein oxidised with 100–200 mM diamide for 30 min at ambient temperature were separated by SDS-PAGE in the absence of reductant. Individual lanes were excised and incubated in 2× SDS-PAGE loading buffer (4% (w/v) SDS, 125 mM Tris–HCl, pH 6.8, 20% (v/v) glycerol, 0.004% (w/v) bromophenol blue) containing 20 mM DTT for 1 h at ambient temperature before being placed horizontally at the top of a second SDS-PAGE gel for separation of proteins in the second dimension. Gels were stained after electrophoresis with 17% colloidal Coomassie (w/v), ammonium sulfate, 34% (v/v) methanol, 3% (v/v) phosphoric acid, 0.1% (w/v) Coomassie brilliant blue G-250) before destaining with 0.05% (v/v) phosphoric acid.

Immunoblotting of gels and detection of Aox subunits was performed as described previously [28,38].

Protein spots were excised from diagonal PAGE gels and destained (50% (v/v) acetonitrile, 25 mM NH_4HCO_3) for 45 min twice each. Gel pieces were dried at 50 °C for 20 min before digestion of the embedded protein overnight with trypsin digestion solution (25 mM NH_4HCO_3, 12.5 μg/ml trypsin, 0.00125% (v/v) trifluoroacetic acid) at 37 °C. Protein was extracted from the gel pieces by incubating for 15 min in 50% (v/v) methanol containing 0.2% (v/v) formic acid for 15 min. The total volume of protein extract was approximately 15 μl. Samples of digested protein were

manually injected into a Q-TOF Mass Spectrometer (Pulsar Q-Star, Applied Biosystems) using Electrospray Ionisation. Doubly charged peptides of interest were selected and fragmented by collision-induced dissociation with N_2 gas, producing MS/MS data. Data analysis was performed (Bio-Analyst software, Applied Biosystems) to determine the amino acid sequence of individual peptides, derived from identification of a, b and y series ions.

3. Results

3.1. Aox is encoded by a small gene family in tomato

Degenerate primers designed against highly conserved residues among plant Aox proteins were used to amplify 170 bp *Aox* gene fragments from tomato genomic DNA [33]. A number of fragments were sequenced and found to encode four different proteins with high similarity to other plant Aox sequences. These gene sequences and the sequence of an Aox gene fragment that had been previously characterised (Holtzapffel, Finnegan and Day, unpublished results), were used to design gene-specific primers. These primers were used to amplify full-length cDNAs for two of the genes from an adaptor-ligated cDNA library prepared from the pericarp of a chilled green tomato fruit.

Similarity searches to previously characterised *Aox* sequences showed that the full-length cDNAs encoded different Aox1-type isozymes [39] and consequently the corresponding genes were named *LeAox1a* and *LeAox1b* (GenBank accession numbers AY034148 and AY034149, respectively). The deduced amino acid sequences of the encoded proteins (Fig. 1) indicate that *LeAox1a* and *LeAox1b* encode proteins of 40 and 36 kDa, respectively, 32.6 and 32.9 kDa after cleavage of the targeting sequence. Both proteins conform to the current model of the Aox active site [15,40]. However, LeAox1b was found to contain a Ser residue at the site of the highly conserved Cys_I residue [15,40], which is necessary for the regulation of the plant Aox enzyme through both redox control and pyruvate activation (see Introduction).

Despite numerous attempts, full-length cDNAs were not obtained for the other two genes originally detected in genomic DNA and it was concluded that these were not expressed in tomato fruit under the conditions used to construct the library. No fruit ESTs for these genes are present in the TIGR tomato data bank. Comparisons with previously characterised Aox sequences show that one of

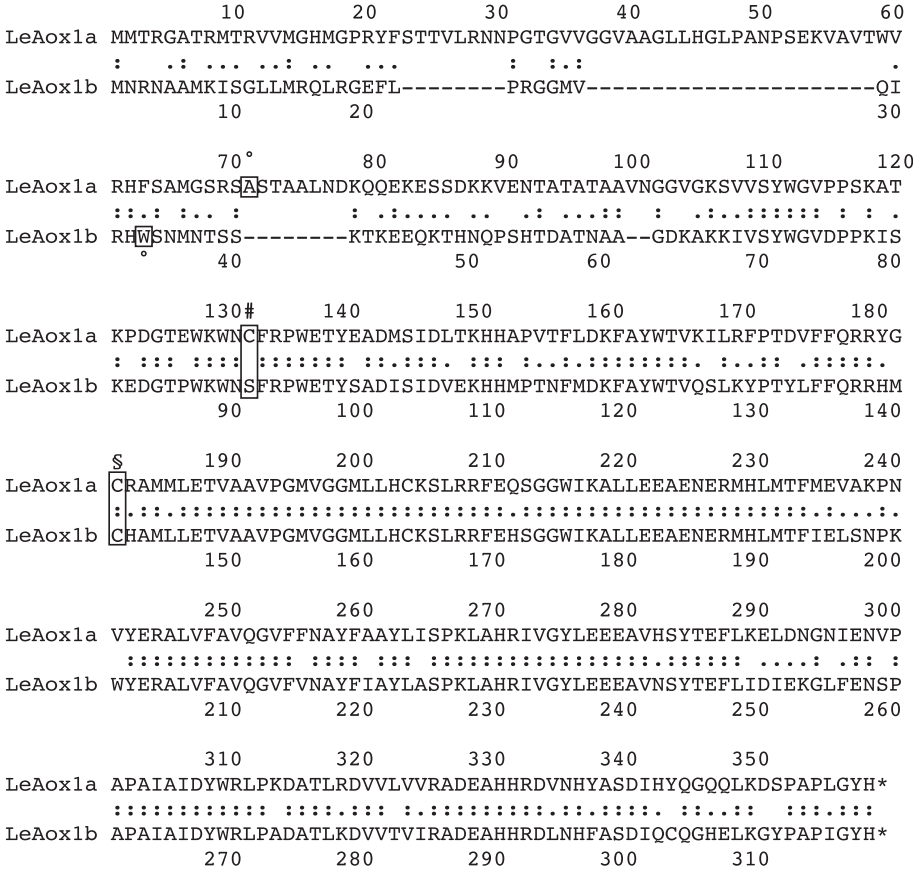

Fig. 1. Alignment of deduced amino acid sequences of LeAox1a and LeAox1b. Amino acid identities (:) and similarities (.) are indicated. The postulated first residue of the mature proteins (°) (assigned after comparison with known mature Aox sequences), the highly conserved Cys_I residue (#) substituted by Ser in LeAox1b and the highly conserved Cys_{II} residue (§) are boxed. Numbering begins at the initiating Met residue for each protein.

the partial genomic fragments belongs to the Aox1 class of proteins and we have termed this LeAox1c (GenBank accession AY324397). A consensus sequence (TC88264) for this gene is present in the TIGR tomato EST data base and was derived from two ESTs isolated from roots. The other partial genomic fragment is from a gene belonging to the Aox2 protein type and we have termed this gene *LeAox2* (GenBank accession AY324396). Two ESTs corresponding to *LeAox2* have been isolated from carpels (EST268259) and roots (EST248494), showing that it is not a pseudogene.

A competitive RT-PCR assay was designed to examine *LeAox1a* and *LeAox1b* expression. Two regions of the *LeAox1a* and *LeAox1b* cDNA sequences showed high sequence identity to one another, allowing the design of a single primer pair that was capable of simultaneously amplifying a 421 bp region from both cDNAs (see Materials and methods). The products originating from *LeAox1a* and *LeAox1b* could be distinguished by unique *Csp*45I and *Rca*I restriction sites, respectively. Using this assay on RNA isolated from a range of tomato tissues, demonstrated that *LeAox1a* transcripts were present in most tomato tissues, including flowers, green fruit, red fruit and chilled green fruit (Fig. 2), but not leaves (data not shown). In contrast, *LeAox1b* transcripts were detected only in RNA samples from chilled green fruit, although very low levels of product were sometimes detected in flower (not shown) and ripe fruit samples (Fig. 2, lane 3). We have shown previously that mitochondria isolated from chilled tomato fruit have extensive Aox activity and two protein bands of 33 and 32 kDa are detected in immunoblots with the AOA antibody [28]. Fresh green fruit and leaves and roots, on the other hand, have only modest Aox activity and only a single, weak protein band at 33 kDa is detected on immunoblots [28,41].

3.2. In vitro translation and import into mitochondria of LeAox1a and LeAox1 precursor proteins

In vitro translation products of *LeAox1a* and *LeAox1b* cDNA transcripts were used for in vitro mitochondrial import experiments to determine the apparent M_r of the mature proteins (Fig. 3). Soybean mitochondria were used in these experiments because we have previously demonstrated that these mitochondria maintain high specificity with in vitro import assays [42,43]. The apparent M_r of the in vitro translation products on SDS-PAGE, 43 kDa for LeAox1a and 36 kDa for LeAox1b (Fig. 3, lane 1), are in good agreement with the sizes predicted from the cDNA sequences. In addition to the precursor proteins, lower M_r bands were present in both precursor samples. We have shown previously that such bands are due to translation initiation at internal methionine codons for other precursor

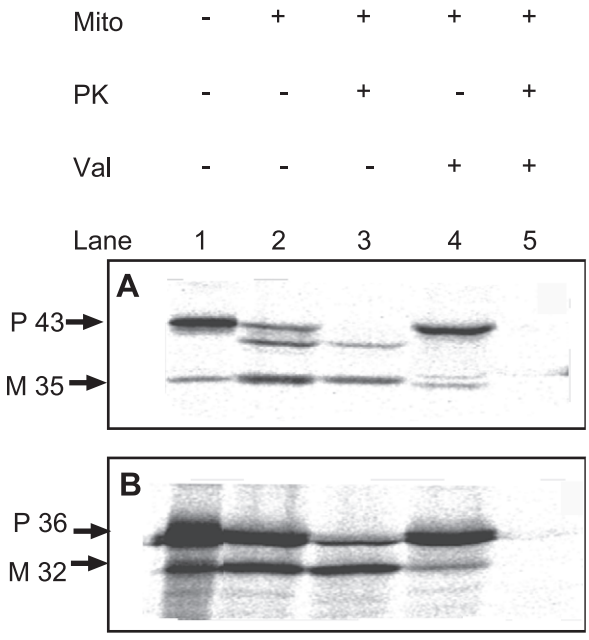

Fig. 3. In vitro import of LeAox1a and LeAox1b into soybean mitochondria. LeAox1a (A) and LeAox1b (B) precursor proteins were ^{35}S-labelled and imported into isolated soybean cotyledon mitochondria as described in Materials and methods. The products of incubating precursor proteins alone (lanes 1): precursor proteins with mitochondria (lanes 2); precursor proteins, mitochondria and proteinase K (PK) (lanes 3); precursor protein, mitochondria, valinomycin (Val), added prior to the addition of the precursor protein (lanes 4) and precursor protein, mitochondria, PK and Val (lanes 5) were separated by SDS-PAGE and visualised by autoradiography. The numbers to the left of the panels indicate approximate molecular mass in kDa. M = mature protein; P = precursor protein.

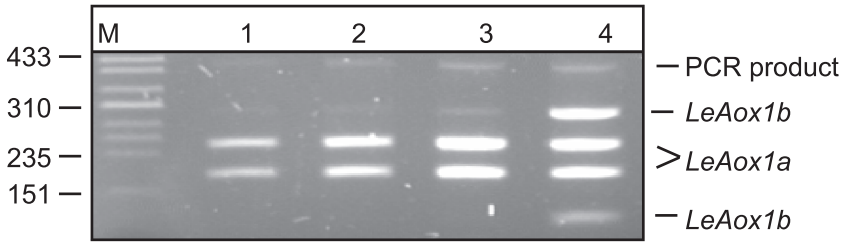

Fig. 2. Detection of *LeAox1a* and *LeAox1b* transcripts by competitive RT-PCR. Approx. 1 μg total RNA isolated from flowers (lane 1), green fruit (lane 2), red fruit (lane 3) and chilled green fruit (lane 4) was used in the RT-PCR assay described in Materials and methods. The 421 bp amplicon was digested with both *Csp*41I and *Rca*I to resolve the products from *LeAox1a* and *1b* cDNAs as indicated on the figure. Undigested 421 bp amplicon can be seen near the top of the gel. Lane M: lambda DNA digested with *Ava*II size markers. The sizes of selected fragments are shown to the left of the figure (bp).

proteins using this system [44,45]. The precursor proteins were incubated with mitochondria under conditions to support protein import. Import was judged by the generation of protease-protected bands that were dependent on the presence of a membrane potential. For the LeAox1a precursor, two protected bands with apparent M_r of 41 and 35 were evident. The latter was similar in size to the additional translation product but it is confidently predicted to be an imported product as it is only PK protected in the presence of a membrane potential (Fig. 3, lane 3 vs. lane 5). The 41 kDa product was only generated when incubated with mitochondria and may represent an import intermediate, as observed with the import of GmAox3 from soybean [46]. Likewise, incubation of the LeAox1b precursor with mitochondria produced a 32 kDa product, which was of similar size to the additional translational product noted above. Again, most of this band was not produced when valinomycin was present and under these conditions was degraded by PK (Fig. 3B, lanes 4 and 5), indicating that it was an imported product. A PK-protected band of ca. 35 kDa was also evident with LeAox1b (Fig. 3B, lane 3), which may represent an import intermediate. Thus both tomato Aox proteins appear to undergo two-stage processing during import.

3.3. LeAox1a but not LeAox1b forms disulfide-linked dimers in the presence of diamide

Oxidation of plant Aox with diamide causes the formation of covalently linked homodimers due to the presence of the highly conserved Cys_I residue [16,17,19]. Diamide was used to oxidise mitochondrial proteins from chilled green tomato fruit, which contain a mixture of 33 and 32 kDa Aox proteins [28], which the mitochondrial import experiments suggest are LeAox1a and LeAox1b, respectively. Diamide treatment caused the preferential oxidation of the 33 kDa protein relative to the 32 kDa protein, but a portion of both proteins remained in the monomeric form (Fig. 4A).

Diagonal PAGE was used to further investigate the dimerisation of Aox proteins in chilled green tomato fruit mitochondria. Using this technique, proteins that can form inter-subunit disulfide bonds have a higher molecular mass during the first dimension of oxidising SDS-PAGE than they do during the second dimension of reducing SDS-PAGE. These proteins appear below the diagonal line that contains unaffected proteins, while proteins forming intra-subunit bonds appear above the diagonal line (Fig. 4B and

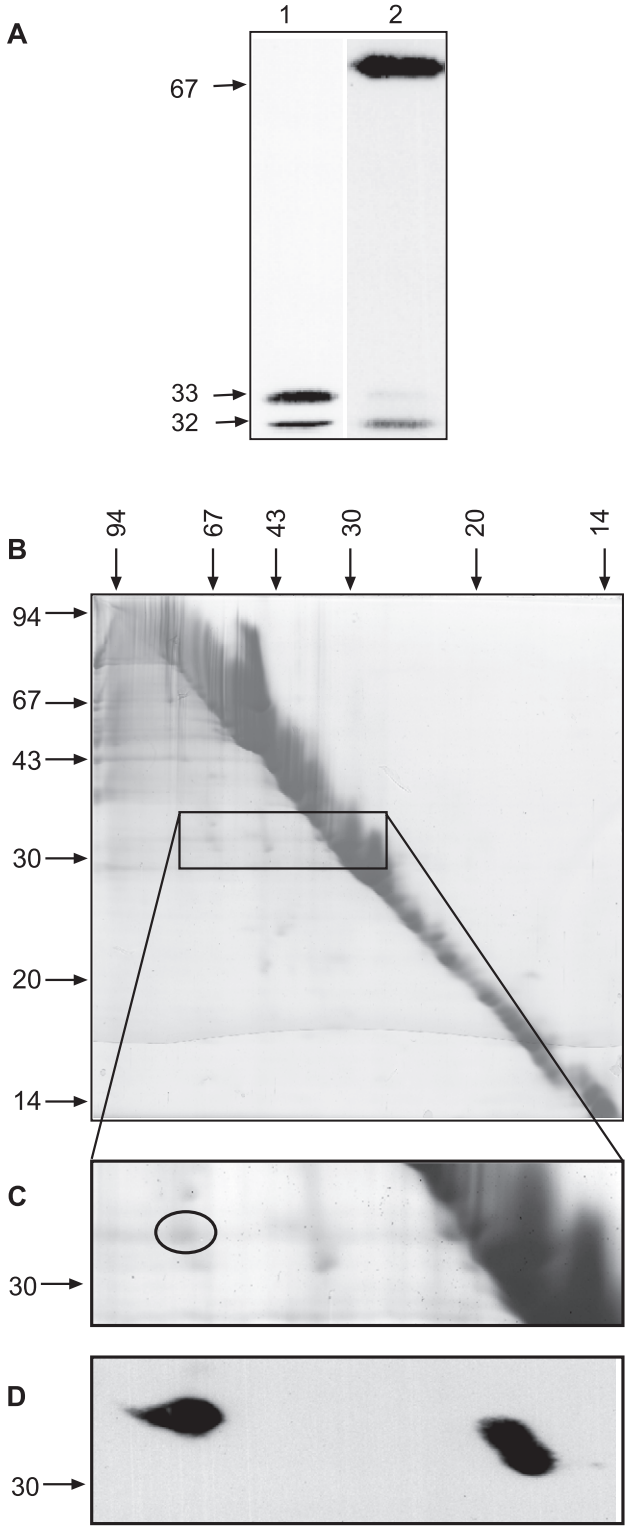

Fig. 4. Separation of redox-sensitive proteins through Diagonal SDS-PAGE. (A) Approx. 40 μg of mitochondrial protein from chilled green tomato fruit were reduced with DTT (lane 1) or oxidised with diamide (lane 2) before separation by SDS-PAGE and immunoblotting with the AOA anti-Aox monoclonal antibody. Approx. M_r of the immunoreactive bands are shown on the left. (B) Approx. 300 μg mitochondrial protein from chilled green tomato fruit was incubated with 200 mM diamide before separation in the first dimension. A lane was excised and incubated in SDS-PAGE buffer containing 20 mM DTT before separation in the second dimension. Proteins were stained with colloidal Coomassie blue. Numbers to the left and above the panel indicate the approximate positions of protein molecular mass markers, sizes in kDa. (C) An enlargement of the 70–35 kDa region is shown with the single spot circled that was found through ESI-Q-TOF MS/MS analysis to contain LeAox1a protein. (D) A 40 μg sample of mitochondrial protein from chilled green tomato mitochondria was treated as described above. After the second dimension, Aox proteins were detected by immunoblotting with the AOA antibody.

Table 1
Results of Q-STAR MS/MS analysis of putative LeAox1a protein from chilled green tomato fruit mitochondria

Peptide charge	Mass/charge ratio of peptide	Actual uncharged peptide mass	Theoretical uncharged peptide mass	Difference (ppm)	Sequence of peptide	Identity of protein
2	1185.6	2369.2822	2369.1730	46	ELDNGMEVPAPAIAIDYWR	*Le*Aox1a
3	709.8	2126.2542	2126.0650	89	no sequence data	*Le*Aox1a
2	653.4	1304.7862	1304.6843	78	SVVSYWGVPPSK	*Le*Aox1a
2	587.3	1172.59	1172.5752	13	ALLEEAENER	Aox

Protein spots were selected based on approximate molecular mass and analysed using an ESI-Q-TOF-MS/MS. Data in the table is from both peptide mass fingerprinting and amino acid sequencing of the most abundant peptides. Peptides were identified as LeAox1a based on the mass of the peptide and/or the sequence determined for it. The actual mass is the mass recorded by the mass spectrometer and is the product of the first two columns minus the value of the charge associated with the peptide. The theoretical peptide mass is the mass determined by the MS Digest program located on the Protein Prospector website (http://www.prospector.ucsf.edu). The peptide identified as "Aox" is present within all known Aox sequences.

C). Immunoblotting was used to determine the positions of the Aox proteins in the second dimension gel. A single protein spot below the diagonal was identified at 33 kDa, while a doublet at 33 and 32 kDa was seen on the diagonal (Fig. 4D). The detection of the 33 kDa Aox protein both below the diagonal line and on the diagonal line indicates that a proportion of the protein in the band visible on 1D SDS-PAGE gels was oxidised to disulfide-linked dimers (Fig. 4D). In contrast, all of the 32 kDa protein was detected on the diagonal, indicating that this protein is incapable of forming disulfide-linked dimers in the presence of diamide. The 33 kDa protein circled in Fig. 4B was excised, digested with trypsin, and sequenced using ESI-Q-TOF MS/MS. It was conclusively identified as LeAox1a (Table 1). It was not possible to directly sequence the 32 kDa Aox band on the diagonal due to the abundance of other proteins at this molecular mass. Neither the 32 nor the 33 kDa protein could be detected in IEF/SDS-PAGE 2D gel separations of tomato mitochondria, thus they could not be directly separated and sequenced in this manner (data not shown). However, the clear identification of the 33 kDa protein as LeAox1a and the non-oxidisible nature of the 32 kDa protein provide further confirmation of the expression of LeAox1b in chilled green tomato mitochondria.

To independently confirm that Cys_I-containing LeAox1a, but not Cys_I-lacking LeAox1b, can be oxidised by diamide, both proteins were expressed separately in *S. cerevisiae*. As shown in Fig. 5, each of the two proteins was detected at its predicted M_r on immunoblots of mitochondria isolated from the transformed yeast cells. However, about 50% of the heterologously expressed LeAox1a was partially degraded, resulting in an additional lower M_r band (Fig. 5, lane 2). Incubation with the oxidant, diamide caused dimerisation of LeAox1a, with two bands detected in the 65–70 kDa region

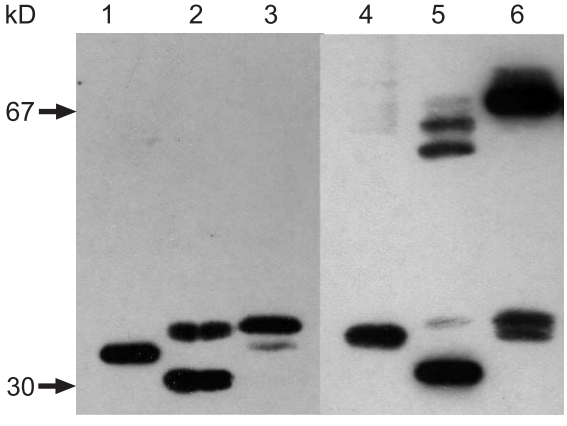

Fig. 5. Immunodetection of Aox proteins in mitochondria. Mitochondria from yeast cells expressing LeAox1b (lanes 1 and 4), yeast cells expressing LeAox1a (lanes 2 and 5) and chilled green tomatoes (lanes 3 and 6). Samples were treated with 20 mM DTT (lanes 1–3) or 200 mM diamide (lanes 3–6) prior to separation. Numbers to the left of the figure indicate the approximate positions of protein molecular mass markers, sizes in kDa.

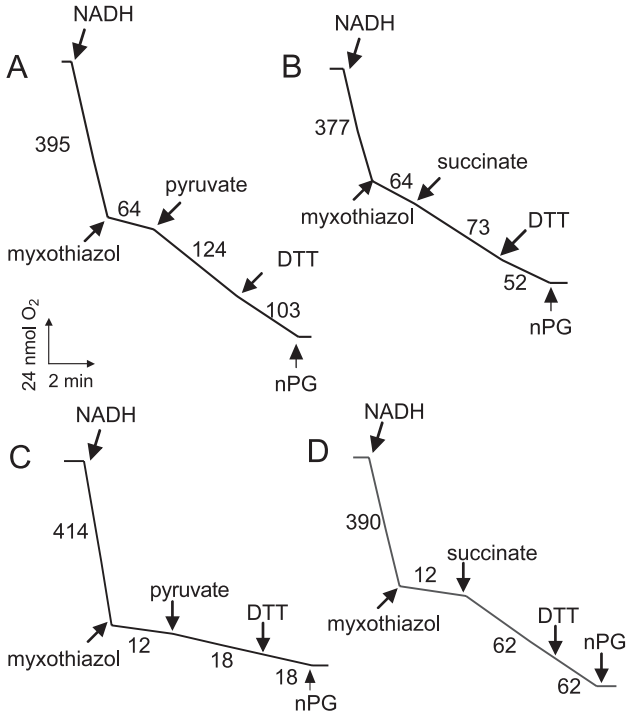

Fig. 6. Oxygen consumption by mitochondria isolated from yeast cells expressing either LeAox1a (A and B) or LeAox1b (C and D). Oxygen uptake was measured as described in Materials and methods, with 0.5 mM ADP included in the reaction medium. Where indicated, the following additions were made to the oxygen electrode chamber: 2 mM NADH; 1.5 μM myxothiazol, 10 mM pyruvate; 10 mM succinate, 5 mM dithiothreitol (DTT), and 50 μM *n*-propylgallate (nPG). Numbers on the traces represent nmol O_2 min^{-1} mg^{-1} protein.

of the blot; these presumably originate from dimerisation of the LeAox1a with its degradation product. LeAox1b was unaffected by diamide, confirming that it is incapable of forming disulfide-linked dimers.

3.4. LeAox1a and LeAox1b are functional Aox proteins with different activation profiles

Although mitochondria purified from chilled green tomato fruit display substantial Aox activity [28], the relative contributions of the two isoforms expressed is difficult to assess. We therefore examined the activities of LeAox1a and LeAox1b expressed in *S. cerevisiae*, to determine their properties. Measurements of oxygen consumption with mitochondria isolated from the transformed yeast showed that both tomato Aox isoforms were active (Fig. 6). The respiration of mitochondria from untransformed yeast cells was completely inhibited by addition of the complex III inhibitor myxothiazol (not shown). The respiration of mitochondria from yeast transformed with *LeAox1a* and *1b* cDNAs, on the other hand, was less sensitive to myxothiazol (Fig. 6), as well as to antimycin A and KCN (not shown). The degree of insensitivity varied from culture to culture, but when Aox was fully activated, the sensitivity was generally 20–25% of state 3 rates with NADH as substrate in mitochondria from cells harvested in late log phase. The myxothiazol-insensitive oxygen uptake was completely inhibited by addition of the Aox inhibitor *n*-propylgallate (Fig. 6). LeAox1a activity was stimulated significantly by the addition of pyruvate (Fig. 6A), as demonstrated with Aox from numerous other plants [9]. Subsequent addition of DTT had little effect, suggesting that the Aox protein was in the reduced form in the isolated mitochondria. This was confirmed by immunoblotting in the absence of added reductant (data not shown). In contrast to LeAox1a, LeAox1b activity was virtually unaffected by pyruvate (Figs. 6C and 7B), but was stimulated by succinate (Figs. 6D and 7B), as found for the Cys_I/Ser substituted GmAox created by site-directed mutagenesis [18,19]. Addition of the succinate dehydrogenase inhibitor malonate did not prevent the stimulation of LeAox1b by succinate (Fig. 7B). Succinate had little effect on LeAox1a activity (Figs. 6B and 7A). Glyoxylate, another activator of Aox in plants [9], stimulated LeAox1a activity as expected but had no significant effect on that of LeAox1b (Fig. 7). This was unexpected, as a Cys_I/Ser substitution in an *Arabidopsis* Aox did not abolish glyoxylate stimulation entirely [19].

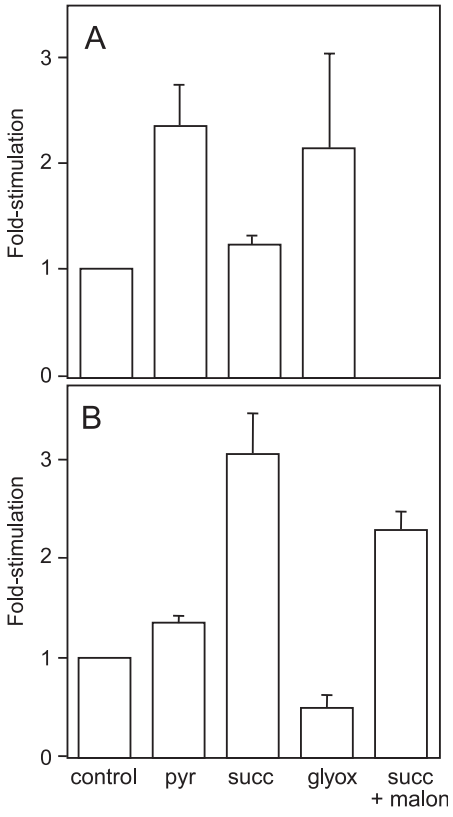

Fig. 7. Effect of various organic acids on alternative oxidase activity in mitochondria isolated from yeast expressing either LeAox1a (A) or LeAox1b (B). Aox activity was measured as oxygen uptake as described in Fig. 6. The organic acids listed on the figure were added at 10 mM. Values shown are mean ± S.E. of 3–10 individual yeast cultures.

4. Discussion

We report here the identification and characterisation of a naturally occurring Cys_I/Ser substituted isoform of Aox, which is expressed in cold-stored tomato fruit. This is the first time that such an isoform has been shown to be expressed in a dicot species. A number of other Cys_I/Ser Aox isoforms have been reported in monocot species ([26,27], TIGR databases) but none of these have yet been shown to be active. In agreement with studies of Aox proteins subjected to site-directed mutagenesis [16–18], LeAox1b cannot form disulfide-linked dimers (Figs. 4 and 5) and therefore cannot be inactivated by oxidation. In addition, LeAox1b is activated by succinate rather than pyruvate (Fig. 6). These data raise the possibility that this is a less regulated form of Aox, which is expressed under conditions of severe stress. The degree to which the Cys_I/Ser substitution affects the regulation of this enzyme in vivo requires further investigation, but since succinate is a common TCA cycle intermediate, and since the enzyme is not subject to redox control, it is likely to be active in the fruit and compete with the cytochrome chain for electrons [14,47]. This would decrease the efficiency of respiration but help to maintain upstream respiration chain components in a more oxidised state, thereby decreasing the potential for active oxygen species generation [2].

Small *Aox* gene families appear to be common throughout the plant kingdom with differential expression of the individual genes under various circumstances [24,27,39]. These isoforms may have subtly different characteristics

from one another, such as ^{18}O discrimination values [19]. There also appears to be a divergence of Aox into two distinct classes in dicots based on amino acid sequences, which may also be expressed differentially in response to developmental, environmental and experimental cues [39]. A member of the Aox1 class (LeAox1a) appears to be generally expressed throughout the tomato plant, although an exhaustive survey was not performed in this study. If, as Considine et al. [39] suggest, Aox1 isoforms are mainly expressed in response to stress, then it is perhaps not surprising that it is only this class of Aox that has been reported to contain mutations of the two regulatory Cys residues. There may be an evolutionary tolerance for such mutations if the less regulated enzyme that results is only expressed under circumstances in which high Aox activity is required.

There have been a number of recent papers investigating the effects of site-directed mutagenesis of the Cys_I residue [16–19]. These have all pointed to the central role of this residue in the regulation of Aox activity. Umbach et al. [19] have shown that such mutations can cause significant differences in the ^{18}O discrimination values of the resulting enzymes suggesting that this residue may interact closely with the active site. The fact that a change from Cys to Ser can dramatically alter the type of organic acid that can interact with and activate the enzyme has implications for the structure of the "pyruvate activation site", which are distinct from the findings that other amino acids can constitutively activate the enzyme when substituted at this position [16,19]. Unlike the SH group of Cys, which can react with pyruvate to form a thiohemiacetal [20], the OH group of serine is not sufficiently reactive to form a strong bond with the succinate molecule and stabilise its interaction with the enzyme. This suggests that there must be other residues that are important for stabilising this interaction with succinate, which may also be important for the interaction of pyruvate. Determining the identity of these residues will add to our knowledge of the structure of the enzyme close to the active site and may lead to further insights into the mechanism of activation by organic acids.

Aox in plants has been shown to exist as either reduced (and active) or oxidised (and inactive) dimers [12]. Expression of multiple isoforms of Aox in a single tissue gives rise to the possibility that heterodimers may be formed [24,48], although this has not been directly investigated to date. The fact that LeAox1a was not able to be fully oxidised in tomato mitochondria may have been due to the formation of heterodimers with LeAox1b, which is not be able to form disulfide-linked dimers. The technique of Diagonal PAGE used here may be useful for investigating whether heterodimers form in species such as *G. max*, which express at least two isoforms of different apparent molecular mass at the same time [24]. Diagonal PAGE also has clear potential as a method for isolating Aox proteins for sequence analysis, which cannot currently be undertaken by methods such as IEF 2D-PAGE due to the general insolubility of denatured plant Aox proteins.

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